Daily Changes in GT1–7 Cell Sensitivity to GnRH Secretagogues That Trigger Ovulation

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Key Words

Gonadotropin-releasing hormone • Kisspeptin • Vasoactive intestinal polypeptide • Reproduction

Abstract

Circadian rhythms in behavior and physiology are orchestrated by a master biological clock located in the suprachiasmatic nucleus (SCN). Circadian oscillations are a cellular property, with 'clock' genes and their protein products forming transcription-translation feedback loops that maintain 24-hour rhythmicity. Although the expression of clock genes is thought to be ubiquitous, the function of local, extra-SCN timing mechanisms remains elusive. We hypothesized that extra-SCN clock genes control local temporal sensitivity to upstream modulatory signals, allowing system-specific processes to be carried out during individual, optimal times of day. To test this possibility, we examined changes in the sensitivity of immortalized GnRH neurons, GT1–7 cells, to timed stimulation by two key neuropeptides thought to trigger ovulation on the afternoon of proestrus, kisspeptin and vasoactive intestinal polypeptide (VIP). We noted a prominent daily rhythm of clock gene expression in this cell line. GT1–7 cells also exhibited daily changes in cellular peptide expression and GnRH secretion in response to kisspeptin and VIP stimulation. These responses occurred without changes in GnRH transcription. These findings are consistent with the notion that GnRH

cells are capable of intrinsic circadian cycles that may be fundamental for coordinating daily changes in sensitivity to signals impacting the reproductive axis.

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Introduction

Optimal brain and body functioning and the maintenance of homeostasis require the precise temporal coordination of numerous hormonal systems [1]. On a daily schedule, these rhythms are controlled by an endogenous circadian timing system that includes both a central, master clock in the suprachiasmatic nucleus (SCN) [2, 3] and subordinate clocks throughout the central nervous system and periphery [4]. In mammals, there are at least two major routes by which the SCN coordinates rhythmic function: hormonal communication [5–8] and autonomic nervous system outputs [9–13]. To achieve system-specific regulatory flexibility, at least some of the downstream signals modulated by the circadian clock vary according to local needs [14].

At the cellular level, circadian oscillations are controlled by a number of autoregulatory transcription-translation feedback loops [15, 16]. The core feedback loop is driven by *Clock: Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like-1 (BMAL-1)*-mediated transcription of three *Period (Per)* gene orthologs and

two *Cryptochrome* (*Cry*) gene homologs. Rhythmic expression of these genes is not only seen in the SCN, but also throughout the central nervous system and periphery, although the coherence of these peripheral oscillators requires a functional master clock [17, 18]. Importantly for the present work, core clock genes and clock-controlled genes are expressed in neurosecretory cells, including GnRH neurons [19, 20], the pituitary gland [21, 22] and a number of peripheral endocrine glands [2, 23, 24], implying a potential intrinsic mechanism of temporal gating which controls hormone secretion.

Given the ability of hormones to have widespread impact throughout the body via the circulatory system, it is important to gain an understanding of circadian endocrine regulation. Daily patterns of hormone secretion are controlled by direct synaptic connections from the SCN to neurosecretory cells in the brain [1]. The SCN also projects multisynaptically to endocrine glands via the autonomic nervous system, although the function of this means of communication remains unspecified [25–28]. For vertebrate reproduction, the precise timing of the hypothalamo-pituitary-gonadal (HPG) axis is crucial [29–31], and direct SCN projections to GnRH neurons have been identified across species [19, 32–34].

In some female rodents, a neural signal arising from the SCN triggers GnRH secretion and induces the preovulatory LH surge on the afternoon of proestrus [35]. Lesioning the SCN or blocking neural output from the SCN eliminates the LH surge [36, 37]. Direct projections from SCN vasoactive intestinal polypeptide (VIP)-expressing cells to GnRH neurons are thought to be critical in LH surge generation [32, 33, 38]. VIP-2 receptors have been found in a subset of GnRH neurons in vivo [39] as well as in the GnRH-releasing cell line, GT1-7 cells [40], further suggesting direct effects of VIP on GnRH neurons. Importantly, manipulations of VIP both in vitro [40, 41] and in vivo lead to marked changes in GnRH and gonadotropin release [34, 42, 43].

In addition to direct SCN regulation of the GnRH system, the SCN projects extensively to the anteroventral periventricular nucleus (AVPV), a brain region associated with the induction of the preovulatory LH surge [44–48]. Kisspeptin, a product of the anti-metastatic *Kiss-1* gene [49] and a potent stimulator of the HPG axis, is highly expressed in the AVPV and has been implicated in the initiation of the LH surge [50]. These findings suggest that kisspeptin may act as an intermediary between the SCN and the reproductive axis as an additional mechanism of ovulatory control. Kisspeptin is believed to communicate directly with the GnRH system via *G-protein-coupled re-*

ceptor 54 (*GPR54*) [49, 51, 52]. A large proportion of GnRH cells co-express *GPR54* mRNA in rodents, further pointing to direct actions of kisspeptin on the GnRH system [53–55]. In accord with these findings, direct kisspeptin application depolarizes GnRH neurons [53]. Finally, gonadotropin release triggered by kisspeptin can be blocked by GnRH antagonists [51, 54, 56], suggesting that kisspeptin does not act at the level of the pituitary.

Because the LH surge occurs during a limited time window and GnRH cells express circadian clock ‘machinery’, we investigated the possibility that the GnRH system exhibits circadian changes in sensitivity to peptides stimulating GnRH release. Such a mechanism would allow local control of ovulatory function at the level of the GnRH system. We examined the response to peptides putatively involved in direct (VIP) and indirect (kisspeptin) circadian actions on the GnRH system. One possibility is that GnRH cells respond differentially across the circadian cycle to both VIP and kisspeptin. This outcome would suggest that the initiation of the surge depends, at least in part, on the timing of responsiveness to direct and indirect SCN signals. Alternatively, it is possible that GnRH cells respond equally throughout the circadian cycle to stimulation, suggesting that the timing of ovulation is controlled by the timed secretion of these peptides by the circadian system. Finally, the GnRH system may respond in a circadian fashion to one of these peptides but not the other, suggesting a combination of these two mechanisms in initiation of the LH surge.

Materials and Methods

Cell Culture

Nucleotide primers and cell culture reagents were obtained from Invitrogen (Carlsbad, Calif., USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, Mo., USA) if not otherwise specified. GT1-7 cells were cultured in 100-mm master plate in Dulbecco’s Modified Eagle’s Medium (DMEM, CA No. 11995-065) and supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. Media was replaced every 2–3 days until confluence was reached. For experiments, the cells utilized were of similar passage (passage 13–20) and confluence (~90%).

Serum Shock and Peptide Treatments

For the serum-shock treatment, regular culture media was replaced by DMEM supplemented with 50% FBS. Two hours after serum shock, cells were switched into serum-free (SF) DMEM [57, 58]. At indicated time points, cells were re-balanced in fresh SF DMEM for 30 min before kisspeptin and VIP treatment to remove the accumulated GnRH resulting from baseline release. Each peptide was then added to experimental wells and incubated for 45 min. As a result, each time point represents a collection of in-

dividual samples stimulated, only once, at various time intervals after the initial serum shock, not a within-subject design with the same cells being stimulated, or samples collected repeatedly. At each time point, the treated individual samples were compared with untreated control wells that were collected for real-time PCR ($n = 3$) or fixed for quantification using a cellular ELISA ($n = 4$).

Reverse Transcription PCR

Reverse transcription PCR (RT-PCR) was performed on mouse brain and GT1-7 cells. Tissue and cells were collected and their total RNA were extracted (RNeasy Mini Kit; Qiagen Inc., Valencia, Calif., USA). 3'-RACE cDNA was then synthesized (SMART cDNA synthesis kit; Clontech Laboratories Inc., Palo Alto, Calif., USA). Touchdown PCR was then conducted using specific primers (sense: 5'-CGTTA TCTGCCGCCA CAAGC-3', and antisense: 5'-TTGCT GTAGG ACATG CAGTG AGCC-3') for mouse *GPR54*: 3 min 95°C initial denaturing followed by 16 touchdown cycles from 68 to 60°C (annealing temperature, decrease 0.5°C every cycle) and continued for another 25 cycles with 60°C annealing temperature.

mRNA Quantification

Cells stimulated at each time point and their relevant untreated controls were harvested and total mRNA was purified. Total RNA was extracted by using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, Calif., USA). 0.5 µg total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif., USA). Real-time PCR was then performed as previously described [59]. Briefly, 0.5 ng/µl cDNA (RNA equivalent) for each sample was used as a template for quantitative PCR using the IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). PCR parameters were: 5 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by melt curve analysis. The raw fluorescent data were analyzed by the Real-time PCR Miner program [59]. The resulting PCR efficiency and fractional cycle number at the threshold (CT) were used for gene quantification. β -Actin was used as an internal control to normalize mRNA levels among samples. The following primers were used for *BMAL-1*: 5'-GCACC AGTGG TGTGG ACTGC AA-3' (sense), 5'-TGCAT ATTCT AACCT TCCAT GAGGG TC-A-3' (antisense); *Period-2*: 5'-ACGGG ACTCT CAGGG CAGTG ACTG-3' (sense), 5'-GTTCT TTGTG TCGCT CAGCT TTG-3' (antisense); *GnRH-1*: 5'-CCAGC CAGCA CT-GGTCTATGG-3' (sense), 5'-CCAGAGCTCCTCGCAGATCC CT-3' (antisense); and β -Actin: 5'-CAGGG TGTGA TGGTG GGAAT GGG-3' (sense), 5'-CCTCG GTGAG CAGCA CAGGG T-3' (antisense).

Enzyme-Linked Immunosorbent Assay

A cellular ELISA was performed for GnRH using β -actin as an internal control. Peptide-treated wells and time-paired controls were used for each time point. Briefly, cells in individual wells were fixed in 4% paraformaldehyde in PBS followed by washes (3×) in dH₂O. PBS with 0.6% hydrogen peroxide (H₂O₂) was then added for 20 min at room temperature (RT) to quench endogenous peroxidase activity. After one wash in PBS, wells were treated with blocking buffer (2% BSA in PBS with 0.1% Triton X-100) for 30 min. Cells were then incubated with the primary antibody solution (rabbit anti-GnRH (CA No. 20075; ImmunoStar, Hudson, Wisc., USA) and mouse anti- β -actin (CA No.

JLA20; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), 1:2,000 diluted in blocking buffer with 0.1% Triton X-100 for 2 h at RT followed by 5 washes in PBS. Biotin-conjugated anti-rabbit secondary antibody solution (1:1,000 in blocking buffer with 0.1% Triton X-100) was then added to wells and incubated for 2 h at RT followed by 5 washes in PBS. Cells were then incubated with streptavidin-conjugated HRP solution (1:1,000 in blocking buffer) for 2 h at RT followed by 5 washes in PBS. Sure-Blue TMB 1-Component Microwell Peroxidase Substrate (Ca No. 52-00-01; KPL, Gaithersburg, Md., USA) was then added and cells were incubated at RT until the reaction was terminated by the addition of 1 N hydrochloric acid (HCl). Resulting OD values were read at 450 nm using a plate reader (Biorad) within ~30 min. The ELISA for released GnRH-1 was performed using a luteinizing hormone-releasing hormone (LHRH) EIA kit (Bachem, Torrance, Calif., USA). All procedures recommended by the manufacturer were followed precisely and the standard curve (0.0064, 0.032, 0.16, 0.8, 4.0, and 20 ng/ml) was parallel to a serially diluted sample, further validating the assay.

Statistics

Data were analyzed by SPSS software using a one-way analysis of variance (ANOVA) to assess main effects of time-course and peptide treatment. To examine significant differences between individual time points and the daily nadir of expression, two-tailed Student's *t* tests were used. For the kisspeptin dose-response curve, two-tailed Student's *t* tests were performed between treated wells and control samples. Results were considered statistically significant when $p < 0.05$.

Results

Clock Genes and GnRH Expression Are Rhythmic in GT1-7 Cells

To confirm the existence of an intrinsic rhythm in clock gene expression in GT1-7 cells, real-time PCR was performed to quantify *Period-2* and *BMAL-1* mRNA across the circadian cycle ($n = 3$ /time point/gene). Both genes exhibited a daily pattern of expression with peaks consistent with a 24-hour rhythm ($p < 0.05$ in each case). As expected, these two rhythms were in anti-phase (i.e., ~12 h out of phase), consistent with the well-established clock regulation model, and further corroborating the measurement of this pattern of expression [60–62]. During the 46-hour monitoring period after serum shock, *BMAL-1* mRNA was at a trough from 13 to 16 h, and recovered between 25 and 37 h, while the mRNA of *Period-2* exhibited the opposite pattern (fig. 1).

Dose-Dependent Stimulation of GnRH Release by Kisspeptin in GT1-7 Cells

By using RT-PCR, we found two alternatively spliced isoforms of *GPR54* expressed in GT1-7 cells (fig. 2; GenBank No. EU532437 and EU879091). To examine wheth-

Fig. 1. Mouse *Period-2* and *BMAL-1* expression exhibit a daily pattern in GT1-7 cells. Fluctuations in mRNA levels after serum shock were quantified by real-time PCR and normalized to mouse β -actin mRNA. Average gene expression is shown as a percentage of the minimal expression during the day. Error bars represent standard errors ($n = 3$). Mouse *Period-2* ($F_{14,44} = 13.407$, $p < 0.001$) and *BMAL-1* ($F_{14,44} = 18.634$, $p < 0.001$) mRNA levels exhibit a ~24-hour pattern in anti-phase to each other. * Significantly greater *Period-2* expression than the lowest value over the course of the sampling period: $p < 0.05$. # Significantly greater *BMAL-1* expression than the lowest value over the course of the sampling period: $p < 0.05$.

Fig. 2. *GPR54* is expressed in GT1-7 cells. Mouse *GPR54* (*mGPR54*) in mouse brain (B) and GT1-7 cells (G) was detected by RT-PCR. Water (W) was used as negative control and β -actin (*mActin*) was used as an internal control.

er kisspeptin stimulates GnRH release in GT1-7 cells, cells were treated for 45 min using a range of kisspeptin doses (1 nM to 10 μ M), at 13 h following serum shock. Kisspeptin-mediated GnRH release was observed at doses ranging from 1 to 10 nM ($p < 0.05$ in each case, $n = 3/$

Fig. 3. Dose-dependent stimulation of GnRH release by kisspeptin treatment in GT1-7 cells. After 13 h of serum starving, GT1-7 cells were treated with kisspeptin (Kiss-1) for 45 min. Error bars represent standard errors ($n = 3$). Kisspeptin significantly (* $p < 0.05$) increased GnRH release at 1 and 10 nM concentrations with no increase seen after higher concentrations (e.g. 1 μ M and 10 μ M) relative to PBS controls.

dose), but was abolished at higher doses (1 and 10 μ M; $p > 0.05$ in each case; fig. 3). As a result, we selected a 5-nM dose of kisspeptin to explore the effects of this peptide on GnRH peptide and mRNA expression, while avoiding potential 'ceiling' or 'floor' effects.

Temporal Sensitivity of GT1-7 Cells in Response to Kisspeptin and VIP

Based on the dose response curve for kisspeptin (fig. 3) and previous reports characterizing the GT1-7 cell response to VIP [40], we chose a 5-nM concentration for kisspeptin and a 0.5-nM concentration for VIP to assess the daily rhythm in responsiveness to both peptides following serum shock. We first examined intracellular GnRH peptide content following kisspeptin treatment. GT1-7 cells showed a robust increase in GnRH peptide following both kisspeptin and VIP treatment 7 h after serum shock ($p < 0.05$ in each case, $n = 4/\text{peptide}$), followed by a general insensitivity to this peptide 13 and 19 h after serum shock ($p > 0.05$ in each case, $n = 4/\text{time point/peptide}$; fig. 4). At 25 h, cells exhibited a small increase in sensitivity to stimulation by kisspeptin and VIP ($p < 0.05$ in both cases, $n = 4/\text{peptide}$).

GnRH mRNA and GnRH peptide release were examined during a 46-hour period. In contrast to measures in

Fig. 4. GT1–7 cells exhibit daily changes in sensitivity to kisspeptin and VIP stimulation. Post-transcriptional regulation of GnRH in GT1–7 cells is responsive to kisspeptin or VIP treatment at various time points up to 25 h after serum shock. Data are presented as the fold increase above untreated samples for each time point. Error bars represent standard errors ($n = 4$). * Significantly greater than the lowest value over the course of the sampling period; $p < 0.05$. Kisspeptin and VIP treatments showed a similar stimulation pattern across the sampling period, with cellular GnRH peptide showing changes in expression level to the stimulation over the course of the sampling period.

peptide expression, GnRH mRNA transcripts were equally affected at all time points following kisspeptin and VIP treatment ($p > 0.05$ in all cases, $n = 3$ /time point/peptide; fig. 5). In contrast, GnRH release in response to kisspeptin (fig. 6A) and VIP (fig. 6B; $p < 0.05$, $n = 3$ /time point/peptide) was differentially affected by time of stimulation. Kisspeptin treatment resulted in an elevation of released GnRH at 28 and 46 h ($p < 0.05$ in each case) with a marginally significant increase 4 h after serum shock. Elevation of GnRH release in response to VIP treatment was observed at 4, 22, and 46 h with maxima up to ~7-fold that of untreated controls ($p < 0.05$ in each case).

Discussion

The present findings suggest that immortalized GnRH cells exhibit intrinsic daily changes in sensitivity to neurochemicals stimulating their activity (fig. 4, 6). Additionally, these studies have uncovered at least two alternatively spliced isoforms of GPR54 in GT1–7 cells (fig. 2),

Fig. 5. GT1–7 cells do not show daily changes in GnRH mRNA following kisspeptin and VIP stimulation. GnRH mRNA levels were detected by real-time PCR after kisspeptin or VIP treatment at various time points up to 46 h after serum shock. Data are presented as the fold increase above untreated samples for each time point. Error bars represent standard errors ($n = 3$). No significant rhythm was detected for GnRH transcription following kisspeptin ($F_{7,23} = 0.962$, $p = 0.490$) or VIP ($F_{7,23} = 1.752$, $p = 0.167$) treatments.

a finding further confirmed in mouse whole-brain cDNA. Other groups have recently reported one isoform of GPR54 in GT1–7 cells [63, 64]. In contrast to the present work, the primers used in these studies do not cover the alternative splicing region of the gene. Administration of kisspeptin promotes GnRH release in a dose-dependent manner with a saturable concentration in GT1–7 cells (fig. 3). Previous reports demonstrated dose-dependent effects of VIP stimulation on GnRH release in this same cell population [40], and we have replicated and extended these earlier findings, showing daily changes in sensitivity to this peptide. Previous studies in perfused GT1–7 cells suggested that rapid transcriptional and translational regulation is not involved in GnRH pulsatility by applying transcriptional and translational inhibitors to these cells [65]. In this study, we investigated GnRH production at the transcriptional level, translational level and secretion level. For both kisspeptin and VIP, no significant differences in GnRH mRNA were detected throughout the day (fig. 5), implying that rapid GnRH transcriptional regulation does not occur in response to peptide stimulation. In contrast, time-dependent responses to kisspeptin and VIP at the translational/post-translational and secretory levels were observed. Togeth-

Fig. 6. GT1-7 cells show daily changes in GnRH release following kisspeptin (5 nM) and VIP (0.5 nM) stimulation. GnRH release in response to kisspeptin or VIP stimulation was monitored at various time points up to 46 h following serum shock. Data are presented as the fold increase above untreated samples for each time point. Error bars represent standard errors ($n = 3$). **a** Kisspeptin treatments initiated maximum GnRH release approximately ev-

ery 20–26 h ($F_{7,23} = 2.707$, $p = 0.047$). **b** Similarly, VIP treatments initiated maximum GnRH release approximately every 22–24 h ($F_{7,23} = 11.675$, $p < 0.001$). A post hoc test (Waller-Duncan test, type I/type II error ratio = 100, significance level = 0.05) was used to compare the means of all time points. Time points not sharing a letter in common are significantly different: $p < 0.05$.

er, these findings indicate that the GT1-7 cells, and perhaps the GnRH system in vivo, exhibit an endogenous time-dependent sensitivity to neurochemical stimulation. However, to confirm a rhythm in sensitivity to GnRH secretagogues, future investigations examining this system across several daily cycles are necessary.

The model system used in the present series of studies allows the investigation of the reproductive neuroendocrine axis uncoupled from the master pacemaker in the SCN. Recent studies suggest that the coordination of subordinate clocks is central for normal physiology [66–69]. As described previously, the peripheral time-keeping system is subordinate to the SCN and requires input from the master clock for its maintenance [18, 62]. Studies using food restriction indicate that peripheral oscillators can become uncoupled from central oscillators when hormonal signals conflict with SCN output [70, 71]. Under normal circumstances, individual cellular clocks from a given system are synchronized to each other, but this synchrony is lost in peripheral clock cell populations in culture when removed from SCN input [18]. In vitro, Chappell et al. [58] found that clock genes in GT1-7 cells oscillate with a circadian period following serum shock, and pulsatile GnRH secretion appears to be regulated by these oscillations. The *Period-2* and *BMAL-1* mRNA patterns in the present studies are consistent with this origi-

nal report. Additional evidence for a circadian mechanism in GT1-7 cells comes from studies in which melatonin treatment leads to increases in cellular melatonin receptor expression, but only at distinct circadian periods [72]. Likewise, the ability of melatonin to inhibit GnRH secretion varies with a circadian period when examined over 48 h in this immortalized population [73]. Our results demonstrate a daily change in sensitivity of GT1-7 cells to kisspeptin and VIP treatment, suggesting the possibility that the intrinsic circadian time-keeping apparatus in extra-SCN cells provides a mechanism for altering local responsiveness to upstream neuromodulators. Future studies using molecular approaches to disrupt the cellular clock are necessary to determine whether these daily changes in sensitivity are mediated by local oscillators in this cell population.

The SCN neuropeptide VIP is a key regulator of the ovulatory cycle in rodents. Administration of VIP antiserum [34] or antisense oligodeoxynucleotides [42] alters the time course and reduces the magnitude of the LH and prolactin surges on the afternoon of proestrus. The present findings indicate that the effects of VIP on the GnRH system depend on the timing of administration. Similar findings have been reported for vasopressin in vivo; vasopressin administration can stimulate the LH surge [74, 75], and its effects have been shown to depend on timing

of administration [75]. Whether vasopressin acts directly on GnRH cells or via an intermediate system remains unclear. One recent study has demonstrated the innervation of AVPV kisspeptin neurons by vasopressin immunoreactive fibers [76], suggesting that this kisspeptin pathway may provide an indirect route between the SCN and the HPG axis [47, 53, 55, 77–79]. A vasopressinergic pathway from the SCN to AVPV kisspeptin cells would allow circadian control of this potent stimulator of reproductive axis function.

Several lines of evidence support a role for AVPV kisspeptin as an integration point for circadian and steroidal signals necessary for initiation of the LH surge. First, a large proportion of kisspeptin cells express estrogen receptor- α , the receptor subtype important for the positive feedback effects of this sex steroid during the surge [45, 80–82]. Additionally, kisspeptin mRNA in the AVPV is increased in a timed manner, with maximum expression on the afternoon of proestrus [50, 83]. Importantly, this expression pattern is dependent on the presence of high concentrations of estrogen [50, 83]. Likewise, kisspeptin neurons exhibit an increase in FOS expression concomitant with the LH surge [50]. Infusion of an anti-kisspeptin antiserum into the pre-optic area of rats blocks the LH surge [83]. In the ewe, kisspeptin can alter the timing of the LH surge and stimulate ovulation in anestrus animals [84]. In anestrus female Siberian hamsters housed in short days, kisspeptin treatment does not lead to an increase in LH, indicating an alteration in GnRH cell sensitivity to this peptide as a potential additional mechanism of ovulatory inhibition during winter [85].

In rodents whose ovulatory cycle is tied to the circadian system, this mechanism ensures that ovulation is coordinated with female sexual motivation and the time of day during which males are seeking a mate. Additionally, estrogen-dependence of this timing mechanism safeguards against the preovulatory LH surge occurring

before the oocyte is fully mature. Together with these previous findings, the present data showing daily changes in GT1–7 cell sensitivity to kisspeptin stimulation suggest that local time-dependent sensitivity of GnRH neurons might be an important additional component of the SCN-HPG and SCN-AVPV-HPG circuits required for proper endocrine timing. Because the present study did not specifically investigate daily changes in the ability of GT1–7 cells to produce GnRH, it is possible that such modifications contribute to observed patterns of expression. Future studies in which GT1–7 cells are stimulated with a neurochemical that consistently causes GnRH release are necessary to examine this possibility.

The present results suggest a mechanism of HPG axis control in which the gating of stimulated GnRH secretion is coordinated locally within the GnRH neuronal network. Whether or not circadian clock genes participate in this rhythm in sensitivity represents an exciting avenue for further investigation. As indicated previously, core circadian clock genes are expressed in neurosecretory cells [87], the pituitary gland [21, 22], and other peripheral endocrine glands [23, 24, 88], suggesting that this mechanism of local temporal control may operate at multiple levels of the HPG axis providing opportunity to explore this novel regulatory system.

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